

## **Rapid Communication:**

### **A silicon nanomembrane platform for the visualization of immune cell trafficking across the human blood-brain barrier under flow**

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**Running title:** Human microfluidic cerebrovascular barrier model

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## **Abstract**

Here we report on the development of a breakthrough **microfluidic human *in vitro* cerebrovascular barrier (CVB)** model featuring stem cell derived brain like endothelial cells (BLECs) and nanoporous silicon nitride (NPN) membranes ( $\mu$ SiM-CVB). The nanoscale thinness of NPN membranes combined with their high permeability and optical transparency make them an ideal scaffold for the assembly of an *in vitro* microfluidic model of the blood-brain barrier (BBB) featuring cellular elements of the neurovascular unit (NVU). Dual-chamber devices divided by NPN membranes yield tight barrier properties in BLECs, and allow an abluminal pericyte-co-culture to be replaced with pericyte-conditioned media. With the benefit of physiological flow and superior imaging quality, the  $\mu$ SiM-CVB platform captures each phase of the multi-step T-cell migration across the BBB in live cell imaging. The small volume of < 100  $\mu$ L of the  $\mu$ SiM-CVB will enable *in vitro* investigations of rare patient derived immune cells with the human BBB. The  $\mu$ SiM-CVB is a breakthrough *in vitro* human BBB model to enable live and high quality imaging of human immune cell interactions with the BBB under physiological flow. We expect it to become a valuable new tool for the study of cerebrovascular pathologies ranging from neuroinflammation to metastatic cancer.

**Keywords:** blood-brain barrier, microfluidics, nanoporous silicon nitride membrane, two-compartmental flow chamber, T-cell migration

## **Introduction**

The endothelial blood-brain barrier (BBB) in central nervous system (CNS) parenchymal microvessels protects the CNS from the constantly changing milieu in the blood stream. Low pinocytotic activity, complex and molecularly distinct tight junctions combined with expression of specific transporters and enzymes make BBB endothelial cells biochemically unique <sup>1</sup>. Importantly, development and maintenance of BBB characteristic in CNS microvascular endothelium is not an intrinsic characteristic but rather relies on the continuous cross-talk with cellular and acellular components of the neurovascular unit <sup>1</sup>. In addition to strictly controlling the movement of molecules across its interfaces the BBB also rules the entry of immune cells and immune mediators into the immune privileged CNS <sup>2</sup>. While under physiological conditions, immune cell entry into the CNS is very low, in CNS inflammatory diseases such as multiple sclerosis (MS) BBB function is impaired and high numbers of immune cells infiltrate the CNS, where they cause inflammation, and demyelination <sup>3</sup>. Therapeutic inhibition of immune cell entry into the CNS has proven beneficial for the treatment of MS <sup>4</sup>. Unfortunately, these therapies come with the rare but severe side effect of progressive multifocal leukoencephalopathy (PML), which is caused by infection of human oligodendrocytes with the JC virus and can thus not be modeled in animals. Furthermore, a number of anti-inflammatory treatments that were highly effective in animal models for MS, have failed in MS trials <sup>5</sup>, underscoring that the autoimmune pathogenesis underlying MS including BBB dysfunction and immune cell entry into the CNS cannot be modelled in their entire complexity in available animal models.

Therefore, there is an unmet need for detailed functional studies employing human disease-relevant tissues and cells. This includes the necessity for human models of the BBB allowing the study of its function and the migration by disease-relevant immune cell subsets across the cellular barrier as a critical step in MS pathogenesis.

Most well-characterized BBB culture models are based on primary brain endothelial cells or brain endothelial cell lines from animal origin (bovine, porcine and murine) <sup>6-8</sup>. Although elegant human *in vitro* BBB models employing primary human brain endothelial cells have been established <sup>9</sup> their availabilities are limited to few laboratories with privileged access to human brain tissue. Thus, human brain endothelial cell lines like the hCMEC/D3 have been established and widely used as *in vitro* models of the

human BBB<sup>10,11</sup>. Although hCMEC/D3 retain morphological and functional characteristics of human brain endothelium, they fail to establish barrier characteristics resembling their tightness observed in BBB *in vivo*<sup>12</sup>. This limits the suitability of hCMEC/D3 for pharmacological, toxicological and functional assays on the human BBB with *in vivo* predictability.

Recently, stem cell sources have demonstrated substantial advantage over other brain endothelial cells sources for BBB modeling given their human origin, stability, scalability, self-renewal and potential to generate syngeneic cellular components of the neurovascular unit<sup>13,14</sup>. While inducible pluripotent stem cell (iPSC) derived *in vitro* BBB models establish very tight barrier properties their immune phenotype has not been well established. We have recently employed cord blood CD34<sup>+</sup> hematopoietic stem cells to differentiate endothelial cells (ECs)<sup>15</sup>. By co-culturing CD34<sup>+</sup>-derived ECs with bovine pericytes we were able to differentiate those ECs into brain-like endothelial cells (BLECs) providing a valuable *in vitro* model for the human BBB<sup>15</sup>. Growing BLECs on conventional cell culture filter inserts in co-culture with pericytes, we and others used both small molecule diffusion and transendothelial electrical resistance to establish that BLECs form tight barriers, show robust expression of BBB signature molecules<sup>15,16</sup> and express adhesion molecules supporting T-cell trafficking across the BBB<sup>17</sup>. In combination with their large-scale availability, BLECs are therefore the ideal culture platform to study the interaction of human immune cells with the human BBB *in vitro*<sup>17</sup>.

Live cell imaging on glass coverslips has been used historically as the means of monitoring immune cell migration across endothelial monolayers *in vitro* under static and flow conditions<sup>18-21</sup>. As glass coverslips are impermeable, they are not suited for establishing *in vitro* BBB models that rely on the cross talk between brain microvascular endothelial cells in a luminal compartment and CNS derived factors in an abluminal compartment<sup>22,23</sup>. On the other hand, the imaging qualities of permeable polymer track-etched (TE) membranes used in filter inserts are too poor to study immune cell interactions with the BBB *in vitro* by live cell imaging. Several studies have also demonstrated that physiological shear forces are key to the multi-step process of T-cells diapedesis<sup>8,24,25</sup>. Thus the ideal platform for the study of dynamic T-cell interactions with the BBB is a membrane-based flow microfluidic system with glass-like imaging quality. Here we report on the development of a novel *in vitro* platform, the  $\mu$ SiM-CVB, for the study of the

interaction of human T cells with human in vitro models of the BBB. The enabling component of this platform is ultrathin nanoporous silicon nitride (NPN), a 50 nm thick ‘nanomembrane’ with a high density ( $10^{10} \text{ cm}^{-2}$ ; 20% porosity) of  $\sim 40 \text{ nm}$  pores<sup>26, 27</sup>. The thinness and high porosity of NPN result in a luminal/abluminal divider with extraordinary small molecule permeability<sup>27, 28</sup> and the sub-optical pore sizes of NPN provide outstanding imaging characteristics. We establish that CD34<sup>+</sup> stem cell derived ECs differentiate into BLECs when cultured on NPN with pericyte-conditioned medium supplied basolaterally, eliminating the confounding effects of subluminal cell cultures on imaging. Finally, we demonstrate the utility of the  $\mu\text{SiM-CVB}$  by capturing each phase of multi-step T-cell migration across the BBB using live cell imaging under physiological flow.

## **Material and Methods**

### **Device Fabrication**

The enabling feature of our engineered devices used in this study is nanoporous silicon nitride (NPN) membranes, also referred to as silicon nanomembranes. The details of the fabrication of these membranes are provided elsewhere<sup>26</sup>, and are commercially available at SiMPore Inc., West Henrietta, NY. The overall dimension of the NPN membrane is  $5.4 \times 5.4 \times 0.3 \text{ mm}^3$  and the permeable freestanding ‘window’ of the chip is  $2 \text{ mm} \times 0.7 \text{ mm} \times 0.05 \text{ mm}$  (50 nm) and have an average pore diameter of 50 nm. The microfluidic device used for static and dynamic assays consist of 2 compartments: apical and basal, separated by the permeable membrane chip in between. The apical and basal fluidic compartments or channels are composed of transparent 300 microns silicone gaskets (Specialty Silicone Fabricators, CA). The different layers of the static and flow devices are shown in Figures 3 and 4 respectively. The channels and the silicon chip are irreversibly bonded to each other by exposing them to UV-Ozone for 10-15 minutes followed with curing at 70°C for 2 hours. The fluidic access ports for these devices are provided by a thick slab of poly dimethyl siloxane (PDMS) (Sylgard 184, Dow Corning). Flow devices have 4 access ports ( $\sim 1 \text{ mm}$  holes punched in the PDMS blocks): 2 for the apical chamber and 2 for the basal chamber, while “Transwell mimetics” only have access ports for the basal chamber. Upper PDMS blocks also have wider punched holes that act as media reservoirs to support cell culture in both types of devices, and are

bonded to the remaining device using same UV-Ozone treatment.

### **CD34<sup>+</sup> cell derived in vitro BBB model**

CD34<sup>+</sup> cells were isolated from human umbilical cord blood (UCB) according to a protocol previously reported <sup>29</sup>. The isolation of CD34<sup>+</sup> cells required the collection of human umbilical cord blood (UCB) from infants. According to the articles L.1243-3 and R.1234-49 of the French legislation regarding the collection, use for Research of tissues and cells coming from human body and its derivatives, organs and blood, UCB collection was approved by the Hospital ethical committee (Béthune Maternity Hospital, Béthune, France). The protocol regarding the use of human tissues and cells was authorized by the French Ministry of Higher Education and Research (CODECOH Number DC2011-1321). CD34<sup>+</sup> cells isolated from the UCB were differentiated in ECM basal medium (ScienCell) supplemented with 20% (v/v) fetal bovine serum (FBS; Life Technologies) and 50 ng/mL of VEGF<sub>165</sub> (PeproTech Inc.), on 1% (w/v) gelatin-coated 24 well plates (2x10<sup>5</sup> cells/well). After 15-20 days ECs are seen in the culture dish. For each experiment, the cells were expanded in 0.2% (w/v) gelatin-coated 100 mm Petri dishes (Corning) in ECM basal medium supplemented with 5% FBS, 1% endothelial cell growth supplement (ECGS; ScienCell) and 50 µg/mL gentamycin (Biochrom AG).

Induction of BBB like characteristics in CD34<sup>+</sup> -derived endothelial cells was achieved by co-culture with bovine pericytes as described before <sup>15</sup> and elaborated on in Supplementary Methods.

### **Endothelial Permeability (Pe) and stimulation**

Endothelial permeability was investigated by measuring the clearance of Lucifer yellow (LY; 20 nM; Life technologies) across the BLECs monolayer exactly as described <sup>15, 30-32</sup> and elaborated in Supplementary Methods. Pro-inflammatory conditions were induced by stimulation with 10 ng/ml human recombinant tumor necrosis factor- $\alpha$  (TNF- $\alpha$ , 210-TA, R&D Systems, Inc.) for 16 to 20h prior to the experiments.

### **Immunofluorescence stainings of BLECs**

For staining in the Transwell® filter inserts, BLECs were fixed in 1% (w/v) formaldehyde and permeabilized

in Triton X-100 (0.1% (w/v) at room temperature (RT). Then filter membranes were blocked for 30 min with skimmed milk 5% (w/v) in PBS. BLEC monolayers were incubated for 1h at RT with antibodies against anti-zonula occludens-1 (ZO-1) or VE-Cadherin. For claudin-5 staining, BLECs were fixed in methanol at -20°C for 30s prior to the staining procedure. For ICAM-1, ICAM-2 and VCAM-1, live BLECs were incubated with 10 µg/ml of the respective antibody for 20 min at 37°C in the culture medium, fixed and permeabilized as described<sup>17</sup>. Secondary antibodies were incubated for 30 min at RT. For F-Actin staining, after Triton permeabilization, cells were incubated with rhodamine-labeled phalloidin for 1h at RT. Nuclei were stained with DAPI at 1 µg/mL.

For BLECs staining on NPN membranes live cells were incubated with 10 µg/ml of anti-ICAM-1, ICAM-2, or VCAM-1 antibody for 20 min at 37°C in the culture medium. Then BLECs were fixed and permeabilized according to the Transwell® inserts protocol. For the claudin-5 and F-actin staining, the same protocol as for BLECs staining on Transwell® filters was applied. After PBS washes secondary antibodies were incubated for 30 min at RT. A detailed protocol is included in Supplementary Methods.

### **Human peripheral blood T cells**

Human CD4<sup>+</sup>CD45RO<sup>+</sup> T helper 1 (Th1) cells were obtained by fluorescence activated cell sorting from the peripheral blood of healthy donors according to their specific expression pattern of chemokine receptors (CXCR3<sup>+</sup>, CCR4<sup>neg</sup> CCR6<sup>neg</sup>) exactly as previously described<sup>33, 34</sup>. Th1 cells were cultured in the presence of interleukin -2 (IL-2, 500U/ml) for a total of 20 days. At the day of the experiment dead cells were removed by a Ficoll gradient (780g, 20 min, 20°C) and Th1 cells were resuspended in migration assay medium (DMEM w/o phenol red, Hepes 25mM, Foetal Bovine Serum 5%, L-glutamine 4mM) at 1x10<sup>6</sup> cells/mL.

### **In vitro live cell imaging**

BLECs were grown to confluency in ECM basal medium supplemented with 5% FBS, 1% ECGs and 50 µg/mL gentamycin for 6 days as described before and stimulated or not 16 h to 20 h prior to the assay with TNF-α at 10 ng/mL (R&D System, 210-TA). Flow was applied by connecting the outlet tubing to a syringe automatically drawn up by a precision pump (Harvard Apparatus, Holliston, MA, USA). The flow

rate was calculated as described in <sup>18</sup>. To allow for the accumulation of sufficient Th1 cells on the BLECs monolayer, Th1 cells were first infused with a flow rate of 0.1 dyne/cm<sup>2</sup> for 4 min (accumulation phase). After the accumulation phase, the tap is switched to infuse migration assay medium only and the flow rate was increased to 1.5 dyne/cm<sup>2</sup> for 30 min (shear phase). T-cell interaction with the BLECs was recorded for 30 min in time lapse mode (1 picture/5 s) using the microscopic equipment that has been described in depth before <sup>18</sup> with the ZEN blue software. Video editing was performed with the software “Adobe After Effect” allowing to export the image sequences as videos running at 30 frames/s as well as to edit the videos to show the different T cell behavior with the BLECs. Fiji software was used to classify the T cell behavioral categories observed on the BLECs under physiological flow (diapedesis, crawling, probing and detaching).

### **Statistical analysis**

Data are shown as the mean  $\pm$  SD. Statistical significance was assessed by Student’s t-test or one- way ANOVA followed by Dunett’s post hoc test. For multiple comparison p-values are indicated in the corresponding figures (\* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001). Statistical analyses comprising calculation of degrees of freedom were done using GraphPad Prism 6 software (Graphpad software, La Jolla, CA, USA).

## **Results**

### **Commercial supports are not suitable for live cell imaging of the human *in vitro* BBB model under physiological flow**

We have previously developed a custom-made flow chamber that allowed us to observe the interaction of immune cells with an *in vitro* model of the mouse BBB established from primary mouse brain microvascular endothelial cells (pMBMECs) by live cell imaging <sup>18</sup>. More recently, we adapted this flow chamber to allow the insertion of commercially available filter membranes <sup>35</sup>. In addition to a significant reduction in imaging quality, we found that the commercial TE membranes could not support the formation of BLEC monolayers or that they immediately resulted in the detachment of the monolayer



with the onset of flow (Supplementary videos 1-4). We found similar negative results with 6 commercially available polymer filters (Supplementary table 1) even with the addition of Matrigel™, laminins, collagens or fibronectin coatings.

A review of experimental microfluidic culture devices described in the literature also suggests that these would not provide ready solutions to our needs. Most generally, these systems are not amenable to live cell observations of T cell/BLEC interactions due to the lack of membrane transparency<sup>36, 37</sup>, and/or due to the fact that the endothelial cell interface is grown vertically<sup>38</sup> or on the luminal surface of tubings<sup>39-41</sup>. Moreover, some of these systems employ large amounts of fluid volume to maintain flow over time<sup>36, 37, 42</sup>, which would limit our ability to study the interaction of a limited number of patient derived T-cell subsets with BLECs. We concluded that both a novel membrane and microfluidic device were needed for live cell imaging of dynamic human T-cell/BBB interactions *in vitro*.

#### **CD34<sup>+</sup>-derived ECs adhere and grow on nanoporous silicon nitride membranes**

We established that CD34<sup>+</sup>-derived ECs readily adhered to and built a confluent monolayer on Matrigel™-coated nanoporous silicon nitride (NPN) under static conditions and that NPN provided glass-like clarity of monolayers in phase contrast microscopy (Fig. 1). NPN is a recently developed ultrathin silicon membrane technology<sup>26</sup> and the third generation of ultrathin porous ‘nanomembranes’ developed using silicon manufacturing techniques<sup>28, 43</sup>. NPN membranes are 50 nm thick with ~20% porosity and a narrow distribution of pores centered around 40 nm. NPN is far more mechanically and chemically robust than our original porous nanocrystalline silicon membranes<sup>26</sup>, and unlike microporous SiO<sub>2</sub> membranes<sup>43, 44</sup>, the nanoscale pores of NPN are invisible to light microscopy. Importantly, we have found that NPN supports vascular endothelial cell adhesion under physiological shear stress, while microporous SiO<sub>2</sub> nanomembranes do not (Supplemental Figure S1), perhaps related to the fact that micron-scale pores disrupt the normal architecture of basement membranes<sup>45</sup>.

#### ***Pericyte co-culture can be replaced by pericyte-conditioned medium to induce BBB characteristics in CD34<sup>+</sup>-derived ECs***

Co-culture of CD34<sup>+</sup>-derived ECs with pericytes is essential for induction of BBB characteristics as complex tight junctions and expression of transporters<sup>15</sup>. However, shadows from out-of-focus pericytes in the basal chamber impair imaging of T-cell/endothelial interactions in a co-culture system. Thus to improve the imaging quality at the endothelial layer, we asked if the pericyte-co-culture could be simply replaced with conditioned medium from the pericyte cultures. To this end, we co-cultured CD34<sup>+</sup>-derived ECs with or without pericytes in the original Transwell® filter co-culture setup for 5 days as positive and negative control, respectively. In parallel, CD34<sup>+</sup>-derived ECs plated on additional matrigel coated Transwell® filter inserts were co-incubated with pericyte-conditioned medium (PCM) from the abluminal (lower compartment) or luminal (upper compartment) side (Fig. 2A,B) and subsequently replaced on a daily basis by alternating between 24h and 48h. In order to obtain the PCM, 2.25x10<sup>4</sup> /well pericytes were seeded into 24-well Costar plates one day prior to seeding the CD34<sup>+</sup>-derived ECs. After the adhesion of the CD34<sup>+</sup>-derived ECs on the Matrigel coated Transwell® filter inserts, the 24 hours PCM was collected from the pericyte cultures and administered to the luminal or abluminal side of the CD34<sup>+</sup>-derived ECs (Fig. 2A,B) and subsequently replaced on a daily basis by alternating between 24h and 48h PCM to ensure an optimal concentration of the pericyte-derived factors (Fig. 2B). After 5 days of culturing the CD34<sup>+</sup>-derived ECs under the different conditions, their barrier characteristics were compared side-by-side by testing the permeability of the EC monolayers for the small molecular weight tracer lucifer yellow (LY) (Fig. 2C). In accordance to our previous findings the monoculture of CD34<sup>+</sup>-derived ECs did not establish a diffusion barrier for LY (Pe LY =  $2.04 \pm 0.48 \times 10^{-3}$  cm/min), while the co-culture with pericytes established brain-like endothelial cells (BLECs) with tight barrier characteristics as shown by the significantly reduced clearance of LY across the EC monolayer (Pe LY =  $0.52 \pm 0.23 \times 10^{-3}$  cm/min; Fig. 2C). PCM applied from the abluminal side of the CD34<sup>+</sup>-derived EC monolayer induced barrier characteristics comparable to those induced by the pericyte-coculture (Pe LY =  $0.55 \pm 0.18 \times 10^{-3}$  cm/min; Fig. 2C). Interestingly, applying PCM from the luminal side failed to induce comparable barrier characteristics underscoring that our *in vitro* model of the BBB appropriately mimics the *in vivo* situation with highly polarized endothelial cells.

To confirm junctional integrity and maturation, EC monolayers were immunostained for the transmembrane adherens and tight junction proteins VE-cadherin and claudin-5, respectively and the

junctional scaffolding protein ZO-1. Abluminal application of PCM induced a junctional architecture in CD34<sup>+</sup>-derived ECs comparable to that induced by co-culture with bovine pericytes<sup>15</sup>. Specifically, the pericyte co-culture or the abluminally administered PCM induced a continuous and more jagged staining of the junctional and scaffolding proteins VE-Cadherin and ZO-1 compared to the monoculture and PCM luminal conditions (Fig. 2D). Moreover, junctional claudin-5 immunostaining was enhanced in BLECs under conditions of pericyte co-culture and in the abluminally administered PCM conditions (Fig. 2D) when compared to controls. F-actin staining revealed the absence of stress fibers underscoring the quiescent nature of the ECs monolayers (Fig. 2D). Also, BLECs in co-culture with pericytes or with abluminal PCM showed comparable upregulation of adhesion molecules such as ICAM-1 and VCAM-1 but not of ICAM-2 upon addition of a pro-inflammatory stimulus eg by addition of TNF- $\alpha$  (Fig. 2E). Taken together, these results show that differentiation of CD34<sup>+</sup>-derived ECs into BLECs can be achieved by applying PCM to the abluminal side.

#### ***CD34<sup>+</sup>-derived ECs establish BBB characteristics when grown on NPN membranes***

In a next step, we asked if PCM induced barrier characteristics in CD34<sup>+</sup>-derived ECs also occurred when the cells were grown on NPN membranes. The silicon chip platform of nanomembranes makes it easy to create customized, tightly bonded microfluidic devices using layer-by-layer assembly and ozone based activation of silicone/PDMS layers<sup>46, 47</sup>. Thus, for these purposes, we fabricated a "Transwell® mimetic", in which a NPN membrane divides an upper and lower compartment in static culture (Fig. 3A). The membrane compartment of this device is open for media and reagent application from the top and promotes easy gas exchange for maintaining physiological pH within the media. The basal compartment can be accessed from fluid ports on the top of the device and is separated from the membrane compartment by the NPN membrane providing the sole area for exchange of factors between compartments. The entire device can be autoclaved and the volume requirements in the upper and lower compartments are only 50  $\mu$ L and 20  $\mu$ L respectively.

In order to induce barrier characteristics in CD34<sup>+</sup>-derived ECs, we followed the same culture procedure as depicted in Fig. 2B. CD34<sup>+</sup>-derived ECs (1x10<sup>5</sup> cells/mL) were seeded on the Matrigel coated NPN

membrane and grown in the absence or presence of PCM applied in the basal compartment (Fig. 3B). CD34<sup>+</sup>-derived ECs were cultured for 5 days replacing the PCM in the basal compartment every 24 hours while alternating the 24h and 48h PCM. Barrier characteristics were tested after 5 days of culture by performing a LY permeability assay (Fig. 3B). The paracellular permeability of EC monolayers was evaluated as described in Supplementary Methods and the PSe was divided by a cell growing area (0,014 cm<sup>2</sup>), in order to generate the endothelial Pe in cm/min. While CD34<sup>+</sup> - derived ECs grown on NPN membranes in monocultures did not establish a tight barrier (Pe LY =  $1.82 \pm 0.60 \times 10^{-3}$  cm/min), in the presence of abluminal PCM, CD34<sup>+</sup> -derived ECs grown on NPN membranes established BLEC characteristics (Pe LY=  $0.39 \pm 0.24 \times 10^{-3}$  cm/min; Fig. 3B), comparable to BLECs grown on Transwell® filter inserts (Fig. 2A). Induction of BLEC properties grown on NPN membranes by abluminal PCM was further confirmed by the junctional localization of VE-cadherin, claudin-5 and ZO-1 (Fig. 3C). Thus, NPN membranes are suitable for the induction of BBB properties by PCM in CD34<sup>+</sup> -derived ECs.

Next, we investigated cell surface expression of adhesion molecules known to mediate T-cell interaction with the BBB on BLECs grown on the NPN membranes. We found constitutive cell surface expression of the adhesion molecule ICAM-2, constitutively expressed in human endothelial cells<sup>48</sup> and to a lesser degree for ICAM-1 but not of VCAM-1 on BLECs (Fig. 3D). To mimic an inflammatory environment, BLECs were stimulated with tumor necrosis factor alpha (TNF-α) (10 ng/mL) for 16 h which resulted in up-regulation of ICAM-1 and VCAM-1, as demonstrated in our previous work<sup>17</sup>. Moreover, the TNF-α stimulation was inducing the formation of F-actin stress fibers as revealed by the immunofluorescence staining (Fig. 3D). Taken together, these results show that our human *in vitro* BBB model is functionally reproduced on NPN and suitable to investigate the role of the signature molecules involved in the T-cell trafficking.

#### ***Establishment of a small scale human in vitro BBB model under flow: Introduction of the μSiM-CVB flow system***

Using the same layer-by-layer fabrication used for open “Transwell® mimetics”, we constructed a closed, two-chamber flow device, which we refer to as “μSiM-CVB flow system” (Fig. 4A). This new device is

composed of several 300  $\mu\text{m}$  thick silicone layers that create the compartments necessary for the apical growth of the CD34<sup>+</sup>-derived ECs and the abluminal application of PCM. The CD34<sup>+</sup>-derived ECs are seeded on the silicon nanomembrane via the top channel that also serves as the flow channel. In addition, reservoirs have been added to the top PDMS block of the device at both ends of each channel. This allows the addition of excess culture medium (200  $\mu\text{L}$  per channel) to prevent evaporation during prolonged cell culture (Fig. 4B). In order to grow the BLECs monolayer, CD34<sup>+</sup>-derived ECs are seeded in the top channel on the silicon nanomembrane (1.4  $\text{mm}^2$ ) at a concentration of  $1 \times 10^6$  cells/mL. Induction of BLECs is achieved by the daily and alternating administration of 24h and 48h PCM for 6 days under static conditions, as established in the “Transwell® mimetic” system. The differentiation of CD34<sup>+</sup> EC to BLECs in the  $\mu\text{SiM-CVB}$  flow system by application of PCM into the lower chamber could be confirmed by the induction of permeability values to LY (Pe LY=  $0.72 \pm 0.13 \times 10^{-3}$  cm/min) comparable to Pe LY values for BLECs induced with abluminal PCM in the standard Transwell® inserts and Transwell® mimetics (Supplemental Figure S2). Furthermore, the junctional localization of VE-cadherin, claudin-5 and ZO-1 confirmed the BLECs junctional maturation by abluminal application of PCM in the  $\mu\text{SiM-CVB}$  flow system (Fig. 4C).

To apply flow on the BLEC monolayer, both ends of the top channels are connected to transparent PVC tubing with the help of 18 gauge straight needles with the tap open to avoid any increased pressure in the chamber. The inlet tubing is then connected to a 3-way tap linked to 2 reservoirs respectively containing the Th1 cell suspension and migration assay medium, and the outlet tubing was connected to a programmable pump.

With this device and scheme, it was possible to superfuse the Th1 cells over the BLEC monolayer and directly image the T cells in phase contrast with a transmitted light inverted microscope equipped with a temperature-controlled chamber (37°C), and to observe the multi-step T-cell migration across the BBB under physiological flow in vitro (scheme in Fig. 4D). To perform the flow experiment, first the Th1 cells were infused in the top channel for 4 min at a low flow rate of 0.1 dyne/cm<sup>2</sup> to allow a first contact with the BLECs (accumulation phase). Then the migration assay medium only was infused and the flow rate was increased to 1.5 dyne/cm<sup>2</sup> to reach a physiological shear for 30 min (shear phase) allowing the study of

post-arrest T-cell behavior on the BLECs monolayer. In order to see if we could image the BLECs at high resolution and keep the focus on the cells for the duration of the flow experiment, a temporal snapshot of the BLECs taken from a video recorded with a 10x magnification was made, showing the first and last frame of a flow movie (Fig. 4E). In order to mimic conditions of the BBB under inflammatory or non-inflammatory conditions, BLECs were pre-treated or not with TNF- $\alpha$  after 5 days of culture with the PCM, when the BBB characteristics (low permeability, mature tight junctions) were established (Fig. 3B,C), 16h before starting the flow experiment. First, we found that the resolution was sufficient to perfectly distinguish each endothelial cell in phase contrast images. We observed that treatment of BLECs with TNF- $\alpha$  induced a more elongated shape of the BLECs before flow application which additionally modified the shape of the BLECs (Fig. 4E), which is in accordance with the induction of F-actin stress fibers as observed with the immunofluorescence staining (Fig. 3D). When starting to apply flow (0 min) the NPN membrane's stiffness kept it perfectly in focus (34min55s) and allowed us to continuously image the BLEC monolayer under physiological flow in the same focal plane. In addition, we ensured that the monolayer stayed perfectly intact and in focus under physiological flow for a time period of at least 30 minutes, which is a prerequisite for following the T cell-BLECs interactions over time.

#### ***Live cell imaging of the interaction of human T cells with BLECs under physiological flow***

In a final step, we employed the  $\mu$ SiM-CVB to study the interaction of human T cells with the human BBB under physiological flow following the same procedure described above. In order to illustrate how we could observe various types of T cell/BLEC interactions with high resolution, we generated temporal snapshots from a video recorded with a 20x magnification where BLECs were stimulated with TNF- $\alpha$  (Fig. 5A). In addition, we also recorded videos with a 10x magnification of T cell-BLEC interactions where BLECs were stimulated (video 2) or not (video 1) with TNF- $\alpha$ . These videos allowed us to have a statistical comparison of the T-cell behavior on the BLEC monolayers in unstimulated and TNF- $\alpha$  stimulated conditions (Fig. 5B), since a higher number of T cells could be tracked compared to higher magnification videos, at the cost of a lower resolution. To analyze the respective T-cell behavior on the BLECs monolayers in a quantitative manner, we performed a visual frame-by-frame offline analysis of the time-

lapse videos. To this end each T cell was categorized into a specific behavioral group. Under both, unstimulated and TNF- $\alpha$  stimulated conditions, the majority of T cells were found to polarize upon arrest (video 1 and 2) and to migrate across the BLEC monolayer with or without prior crawling on the BLECs. These were categorized under “diapedesis” (Fig. 5A). T cells that crawled on the surface of the BLECs for the entire observation time were categorized as “crawling” (video 1 and 2, Fig. 5A). One additional behavior observed was T cells that remained stationary without displacing beyond a distance exceeding their own diameter and presenting dynamic cellular protrusions and thus “probing” the BLEC monolayer (video 1 and 2, Fig. 5A). Also, some T cells were found to quickly “detach” from the BLECs monolayer when the flow rate increased at the beginning of the shear phase (video 2). In general, we observed a higher number of T cells arresting on the TNF- $\alpha$  stimulated BLECs versus the non-stimulated BLECs monolayers resulting in an increased number of T cells interacting with the TNF- $\alpha$  stimulated BLECs in each T cell behavioral category (Fig. 5B). Overall, the TNF- $\alpha$  stimulation favored T cell-BLECs interactions without modulating their behavior among categories. This observation is in accordance with the upregulated cell surface expression of ICAM-1 and VCAM-1 previously shown (Fig. 3D), suggesting that upon TNF- $\alpha$  stimulation, the upregulation of adhesion molecules could explain the significantly higher number of Th1 cells found to arrest on BLECs under physiological flow, ~4 min after starting to infuse T cells and just after the accumulation phase (Fig. 5B). Taken together, our novel  $\mu$ SiM-CVB flow system allows for detailed observation of the multi-step T cell extravasation across a human model of the BBB at a very small scale suitable for employing rare patient derived immune cells.

## **Discussion**

The development of an *in vitro* platform to study the migration of immune cells across the BBB in the context of human CNS disorders has provided many challenges. We began addressing these challenges by first establishing a novel human *in vitro* model of the BBB from human ECs derived from cord blood CD34<sup>+</sup> hematopoietic stem cells <sup>15</sup>. We demonstrated that when co-cultured with pericytes, CD34<sup>+</sup> EC

differentiate into brain-like endothelial cells (BLECs) establishing low permeability barriers and mature tight junctions. Due to the general availability of cord blood stem cells, BLECs can be widely adopted to study molecular mechanisms in pathologies implicating the BBB such as neurodegenerative or neuroinflammatory disorders (e.g. Alzheimer's disease, multiple sclerosis), stroke and traumatic brain injury, infectious processes and inflammatory pain.

While we have previously used BLECs to study immune cell extravasation and cancer metastatic cells across the BBB in static culture conditions <sup>16, 17, 49</sup>, the application of physiological flow is needed for more physiological observations <sup>8, 25</sup>. The visualization of the multi-step immune cell extravasation across the vascular wall requires a testing platform compatible with transmitted light microscopy, preferably phase contrast. The desire for flow and live-cell imaging has compelled us and others to use flow chambers with endothelial cells cultured on glass coverslips, as the track-etched membranes used in traditional two-chamber filter devices are notoriously bad for imaging under transmitted light <sup>44</sup>. This choice however, introduces new complications as non-permeable substrates are not physiological and are thought to compromise the polarization of barrier cells due to the lack of basal transport <sup>50</sup>. In addition, these flow chambers are not suitable for *in vitro* BBB models that rely on the continuous and polarized cross-talk of the brain endothelial cells with cellular or acellular elements of the neurovascular unit, such as pericytes or astrocytes and CNS derived factors, respectively <sup>51, 52</sup>.

The previous decade has witnessed the emergence of customized microsystems for 'organ-on-chip' models or 'microphysiological systems' (MPS) <sup>53</sup>. While a number of these efforts have focused on BBB <sup>36-39, 41</sup>, only Walter and colleagues <sup>54</sup> addressed the need for membrane transparency. Their solution was to employ 'transparent' track etched membranes that have 100 fold fewer pores than conventional materials ( $10^6$  pores/cm<sup>2</sup> vs  $10^8$  pores/cm<sup>2</sup>) and are more than twice as thick (23  $\mu$ m vs 6 – 10  $\mu$ m). While these membrane modifications diminish the confounding influence of pores on light transmission and enabled clear phase images of monolayers, they also dramatically reduce the diffusive permeability of the membranes by more than two orders of magnitude. Indeed, we calculated the small molecule permeability of the low porosity TE membranes used by Walter et al. to be  $10^{-5}$  cm/s, a value lower than some of the permeabilities we report here for luciferase yellow transport across BLEC monolayers. Thus,



these low porosity TE membranes improve imaging, but can be rate limiting for small molecule transport if used in a two chambers system.

We addressed the need for optical transparency by building the first *in vitro* barrier models featuring nanoporous silicon nitride (NPN) <sup>26</sup>. NPN is a durable and highly manufacturable ‘silicon nanomembrane,’ a family of ultrathin free-standing porous membranes produced by silicon microfabrication methods <sup>28, 43, 55</sup>. NPN membranes are 50 nm thick NPN with ~ 40 nm pores and a density of  $10^{10}$  pores/cm<sup>2</sup> <sup>26</sup>. The small molecule diffusive permeability of these membranes is a remarkable ~ 0.1 cm/s <sup>56</sup>, a value so high that is indistinguishable from free diffusion through water in practical assays <sup>27</sup>. For our purposes, NPN is also superior to the microporous SiO<sub>2</sub> nanomembranes previously used to visualize direct HUVEC/ADSC co-cultures <sup>43</sup> because: 1) The sub-optical pore sizes of NPN give a feature-free background for high quality visualizing immune cell/endothelial interactions; 2) Endothelial cell adhesion to NPN is excellent even under high levels of shear stress that readily strips endothelial cells from microporous SiO<sub>2</sub> (Figure S1). In addition to their extraordinary permeability, NPN membranes were superior to any commercially available membrane we tested with respect to their optical characteristics and adhesive properties. Indeed, the CD34<sup>+</sup> ECs grew perfectly on the NPN membrane reaching confluency 24h after seeding and building a dense monolayer after 6 days of culture that allowed maintenance of a BLEC monolayer under physiological flow. Because the silicon platform enables the facile construction of dual compartment culture devices, we readily adapted the co-culture based human *in vitro* BBB model requiring co-culture of CD34<sup>+</sup> ECs with pericytes <sup>15</sup>. Successful replacement of the pericyte co-culture with PCM in the basal compartment further advanced the *in vitro* BBB model by eliminating contributions from out-of-focus cells when imaging the endothelial layer. Using PCM instead of the standard co-culture with pericytes revealed only one minor difference with respect to a lack of upregulated expression of ICAM-2 upon stimulation of BLECs with TNF- $\alpha$ . However, since ICAM-1 is the major and high affinity ligand for LFA-1 on T cells, this result does not invalidate our model to study immune cell trafficking. Moreover, regarding the difference in the model’s characteristics, we noticed that after the transfer of the BLECs from the “Transwell® mimetic” to the  $\mu$ SiM-CVB flow system, the claudin-5 staining appeared more jagged, which could be a sign of a different junctional maturation. Since we measured comparable permeability values of the BLEC

monolayer in the two devices, it seems that this does not have a significant impact on the paracellular tightness that could modify immune cell interactions with the BLECs. However, this observation may become important when adapting this *in vitro* BBB model for small molecule permeability screenings. The next step, which was to apply flow in our  $\mu$ SiM-CVB flow system, took full advantage of the properties of the NPN material that help distinguish it from all commercial membrane materials. While its nanoscale thinness gives NPN high permeability and makes it excellent for live cell imaging, we also found that the ceramic-like membrane is still sufficiently stiff that it remains in focus with the onset of flow and throughout more than 30 minutes of continuous recording. The optical imaging quality of T-cell interactions observed on the BLECs monolayer using the novel  $\mu$ SiM-CVB flow system were indistinguishable from those on glass<sup>32, 57, 58</sup>. The  $\mu$ SiM-CVB flow system traps T cells below the BLEC monolayer, which does not allow for easy T cell collection and may have consequences for the experimental outcomes. Artificial T cell accumulation e.g. when investigating chemokine gradients can be avoided by modifying the T-cell numbers superfused over the BLEC monolayer and the time of recording.

Despite current trends toward increasing complexity in the development of microphysiological systems, it was not our goal to achieve an anatomical mimetic of the human BBB by including all the cellular and acellular elements of the neurovascular unit. Instead we focused on addressing a specific unmet need in the CVB research community: an *in vitro* platform that enables the observation of live human immune cells interacting with a well-characterized human BBB model under flow. The observations of T-cell arrest, crawling on, and diapedesis across the BLECs monolayer under physiological flow as well as the observed influence of the inflammatory status of BLECs on these interactions, validates the  $\mu$ SiM-CVB for these purposes. Simplicity, such as the elimination of the need for co-culture, is one benefit of our platform. Moreover, the volumes of the apical channels of both the “Transwell® mimetic” and the  $\mu$ SiM-CVB flow system are less than 20  $\mu$ L, a feature that will eventually allow the use of small samples of body fluids or cells obtained from patients for personalized medicine. Overall, we expect the  $\mu$ SiM-CVB platform to open new opportunities to define novel therapeutic targets for neurological disorders ranging from neuroinflammation and neurodegeneration to tumor metastasis and for adaptation to investigate

other cellular barriers.

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### **Author's contributions**

Britta Engelhardt: Conceptualization; Resources; Supervision; Funding acquisition; Project administration; Writing—review and editing. Adrien Mossu: Data curation; Formal analysis; Investigation; Methodology; Writing—original draft. Maria Rosito: Data curation; Formal analysis; Investigation; Methodology; Writing—original draft. Tejas Khire: Data curation; Formal analysis; Investigation; Methodology; Writing—original draft. Hung Chung: Data curation; Methodology. Hideaki Nishihara: Data curation; Formal analysis; Investigation; Methodology. Isabelle Gruber: Data curation; Formal analysis; Investigation; Methodology. Emma Luke: Methodology. Lucie Dehouck: Resources; Methodology. Federica Sallusto: Resources; Methodology. Fabien Gosselet: Resources; Methodology; Writing—review and editing. James McGrath: Conceptualization; Supervision; Funding acquisition; Project administration; Writing—review and editing.

### **Conflict of interests**

J.L.M. declares a competing financial interest as a co-founder and equity holder of SiMPore Inc., a commercial manufacturer of NPN and other silicon-based membrane materials.

Supplementary material for this paper can be found at  
<http://jcbfm.sagepub.com/content/by/supplemental-data>

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## **Figure legends**

### **Figure 1. CD34<sup>+</sup> - derived ECs grow to confluent monolayer on the nanoporous silicon nitride membrane.**

(A) Schematic of the nanoporous silicon nitride membrane used for the cell culture experiments. The free-standing membrane region is a 2 mm x 0.7 mm window in the center of a 5.4 mm x 5.4 mm membrane 'chip'. (B) The cross-sectional (A-A) schematic of the membrane. The bulk substrate consists of 300 microns of crystalline silicon, which is etched at the center to remove the bulk leaving a free standing 50 nm thin silicon nitride membrane. The manufacturing of these membranes was described before<sup>26</sup>. (C) Optical micrograph of the membrane chip. (D) Scanning electron micrograph of the porous membrane demonstrating the ultrathin nature and porous structure of the silicon nitride membrane (> 20% porosity in this example). (E) Phase contrast pictures of CD34<sup>+</sup> -derived ECs adhering and growing to confluent monolayer on the NPN membranes from day 0 until day 5 after plating. The CD34<sup>+</sup> ECs adhere on the NPN membrane after 1 to 2 hours and start to grow to form a confluent monolayer between 1 and 2 days of culture. Cellular density increases until day 5. Scale bar, 100  $\mu$ m.

### **Figure 2. Induction of BBB characteristics in CD34<sup>+</sup> ECs by pericyte-conditioned medium**

(A) Scheme of the different culture conditions used to test the BBB induction (B) Timeline of experimental procedures: scheme of the PCM production and luminal or abluminal administration to the CD34<sup>+</sup>-derived ECs followed by Lucifer Yellow (LY) permeability assay, immunofluorescence stainings and live cell imaging under physiological flow. (C) Lucifer Yellow (LY) permeability assay: the permeability was measured across monocultures of CD34<sup>+</sup>-derived ECs (monoculture), CD34<sup>+</sup> -derived ECs co-cultured with pericytes (co-culture), or pericyte-conditioned medium at the abluminal (PCM abluminal) or luminal (PCM luminal) side. Bars show means  $\pm$  SD of n= 3 independent experiments performed at least in duplicate for each condition. Statistical analysis: one-way ANOVA followed by Dunnett's multiple comparison test \*\*\*\* p < 0.0001. (D) Immunofluorescence stainings for the junctional proteins VE-cadherin (red), ZO-1 (green), claudin-5 (green) and for F-actin (red) in CD34<sup>+</sup> -derived ECs grown in monoculture, in co-culture with pericytes or with PCM administered at the abluminal or luminal side are shown. The pictures show a more



jagged VE-cadherin and ZO-1 fluorescent staining in the co-culture and PCM conditions (arrows). The claudin 5 staining is also more important and continuous in the co-culture and PCM abluminal conditions (arrows). Scale bar, 50  $\mu$ m. (E) Immunofluorescence staining for ICAM-1 and ICAM-2 (red), VCAM-1 (green), on CD34<sup>+</sup>-derived ECs grown in co-culture with pericytes or with PCM administered at the abluminal side, non-stimulated (NS) or stimulated with TNF- $\alpha$  for 16h. Scale bar, 20  $\mu$ m.

**Figure 3. CD34<sup>+</sup>-derived ECs grown on nanoporous silicon nitride membranes establish BLECs characteristics.**

(A) Design of a “Transwell mimetic” device developed to culture CD34<sup>+</sup>-derived ECs in the membrane compartment of the NPN membrane, in close proximity to the pericyte-conditioned medium (PCM) in the basal compartment. The device is constructed from poly dimethyl siloxane (PDMS) and silicone gaskets sandwiched together using UV-ozone bonding. Note the transparent nature of the whole device in general, and specifically of the silicon membrane used to support cell growth. The schematic is shown on the right (drawn not to scale). Molecular tracers can be added to the membrane compartment and collected from the basal compartment for fluorometric analysis. (B) The permeability Lucifer Yellow (PeLY) was measured across monolayers of CD34<sup>+</sup>-derived ECs grown alone (monoculture) and CD34<sup>+</sup>-derived ECs cultured in the presence of PCM in the abluminal compartment. The values show mean  $\pm$  SD of 11 customized devices for the CD34<sup>+</sup>-derived ECs mono-cultures and 12 devices for the CD34<sup>+</sup> ECs cultured in the presence of abluminal PCM. Statistical analysis: Student’s t-test \*\*\*\* $p < 0.0001$ . (C)

Immunofluorescence staining on BLECs, induced by PCM in the basal compartment, for the junctional markers ZO-1 (green), VE-cadherin (green) and claudin-5 (green). Each staining is representative of 2 independent experiment performed on 2 distinct devices, Scale bar, 20  $\mu$ m. (D) Immunofluorescence staining for ICAM-1 and ICAM-2 (red), VCAM-1 (green), and F-actin (red) on BLECs unstimulated (NS) or stimulated with TNF- $\alpha$  for 16h. Each staining is representative of 2 independent experiment performed on 2 distinct devices. Scale bar, 20  $\mu$ m.

**Figure 4: Assembly of the  $\mu$ SiM-CVB flow system**

(A) Photograph and (B) exploded view of the assembled  $\mu$ SiM-CVB flow system. Note the optical transparency of the assembled device components. The supporting layer of the device is a thin #1.5 glass cover slip. All the silicone layers (blue/green/teal) are 300 microns thick.

'Top' and 'Bottom' channels perfuse media, while the 'Top Connecting Layer' and 'Bottom Connecting Layer' demarcate the top and bottom compartments respectively. The 'Membrane Layer' provides a supporting framework for the membrane. The topmost PDMS reservoirs store the excess media and are exposed to the outside-incubator environment for gas exchange. Similarly, all the other device components are all gas-permeable and are sterilized by autoclaving. During flow set-up, the 18 gauge needles fitted with silicone tubings are inserted in the 4 holes in the Top Connecting Layer. (C)

Immunofluorescence staining on BLECs grown in the  $\mu$ SiM-CVB flow system, induced by PCM in the basal compartment, for the junctional markers ZO-1 (green), VE-cadherin (green) and claudin-5 (green). Each staining is representative of 2 independent experiment performed on 2 distinct devices, Scale bar, 20  $\mu$ m.

(D) Schematic of the settings for live cell imaging of the T cell/BLEC interactions under flow. T cells are perfused on the apical (top) side of the device, and migrate in a multi-step process across the BLEC monolayer. T cells can also crawl in the subendothelial layer above the membrane. The 50 nm pores in the silicon membrane prevent their passage into the basal (bottom) compartment of the device. The working distance of the device (distance between microscope objective and T cells) is 1.180 mm. (E) A temporal snapshot to illustrate the resolution and focusing-stability under flow from two videos recorded at 10x magnification (EC Plan-Neofluar 10X/0.30 Ph1). BLECs were cultured and TNF-activated as described in the main text. The pictures show the BLECs to illustrate image clarity and the stability of focus from the start (00min) to the end (34min55s) of the recording session under flow. The arrows show area where the BLECs are elongated after stimulation with TNF- $\alpha$ . The time is displayed on the top left of the video (min:sec format). Scale = 50  $\mu$ m.

**Figure 5. *in vitro* live cell imaging of the T cell-BBB interactions under flow**

(A) A temporal snapshot illustrating the different T cell-BLECs interactions has been made with several frames taken from a video recorded with a 20x magnification (LD Plan-Neofluar 20X/0.4 Korr Ph2). The

CD34<sup>+</sup> ECs were cultured in the top channel of the flow chamber with the PCM in the bottom channel for 6 days and the CD34<sup>+</sup> ECs were stimulated with TNF (10 ng/mL) 16h before starting to record the video. Th1 cells are allowed to accumulate on the BLECs monolayer at a low flow rate of 0.1 dyne/cm<sup>2</sup> for 4 min from the first frame after the first Th1 cells appear in the field of view, then the flow rate was set to 1.5 dyne/cm<sup>2</sup> for 30 min (shear phase). Each row is showing a specific behavior of the Th1 cells with the BLECs with the example of 2 different cells one highlighted with a blue arrow and the other one with a yellow arrow. The first row illustrates the diapedesis event where T cells start to arrest (1min20s) and then polarize and firmly adhere (5min) to the BLECs. Then the T cells start to transmigrate through the BLECs monolayer (5min35s) and take more (23min, blue arrow) or less (7min45s, yellow arrow) time to complete diapedesis. The pore size of 50 nm prevents the leukocytes from crossing the silicon membrane, and cells are trapped in the subendothelial spaces and can still be followed and identified by their darker gray shape in comparison of the BLECs monolayer (23min). The second row illustrates the crawling event where T cells first arrest (1min20s) and then polarize and firmly adhere (3min) to the BLECs. Then the T cells start to form a leading edge with a lot of small protrusions (5min25s, blue arrow and 13min45s, yellow arrow) and actively move on the BLECs against the direction of the flow until they even disappear from the field of view (13min45s, blue arrow and 29min50s, yellow arrow). The third row illustrates the probing event where T cells first arrest (1min20s) and then polarize and firmly adhere (4min40s) to the BLECs. Then the T cells remained stationary without displacing beyond a distance exceeding their own diameter and presenting the ability to greatly modulate their shape, sending a lot of dynamic cellular protrusions around them (from 7min30s to 26min). The red arrow shows the direction of flow and the time is displayed on the top left of the video (min:sec format). Scale = 20  $\mu$ m.

(B) Analysis of the arrest and postarrest T-cell interactions with NS and TNF- $\alpha$  stimulated BLECs in the field of view of the videos recorded with a 10x magnification (example in video 1 and 2) under physiological flow (1.5 dyne/cm<sup>2</sup>) for 30 min. T cells remaining arrested to the BLECs monolayer were quantified (total) at the end of the accumulation phase (0.1 dyne/cm<sup>2</sup> for 4 min from the time the first T cells appear in the field of view). Then post-arrest T cell behavior on the BLECs monolayer under flow was analyzed such as each T cell was assigned to one of 4 categories as follows: diapedesis, crawling, probing and detaching.

Values are means  $\pm$  SD for 10 assays for each condition. Statistical analysis: t-test \*  $p < 0.05$ , \*\*  $p < 0.01$ ,  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .